Molecular Identification of Entamoeba histolytica Parasite by Using Actin and Amebapore-A Genes
التشخيص الجزيئي لطفيلي Entamoeba histolytica بأستخدام المورثين Actin و Amebapore-A

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الخلاصة

الهدف: أجريت الدراسة الحالية في محافظة النجف للفترة من نيسان 2013 ولغاية تشرين الثاني 2013 ، **والتي** تهدف الى التعرف على طفيلي الأميبا الحالة للنسيج *Entamoeba histolytica* **من خلال** التحري عن جينات *Actin* و *Amebapore-A* في عينات البراز **بأستخدام** تقنية التفاعل التسلسلي لأنزيم البلمرة Real-Time PCR.

المنهجية: تضمنت **الدراسة** ، **جمع** (104) عينة براز من المرضى الذين يعانون من الإسهال **والوافدين** الى مستشفى مدينة الصدر الطبية ومستشفى الزهراء للولادة **والأطفال ومستشفى المناذرة ومستشفى السجاد** .

النتائج: كشفت نتائج فحص التفاعل التسلسلي لأنزيم البلمرة Real-Time PCR ، وجود طفيلي الأميبا الحالة للنسيج في (25) عينة براز من أصل (104) عينة **وبنسبة** (24%).

الأستنتاجات: على ضوء النتائج التي تم الحصول عليها من خلال هذه الدراسة يستنتج بأن لتقنية Real-Time PCR كفاءة كبيرة وسريعة في تشخيص طفيلي الأميبا الحالة للنسيج في عينات البراز.

التوصيات: دراسة التعبير الجيني Gene Expression لجينات *Actin* و *Amebapore-A* النوعية بطفيلي الأميبا الحالة للنسيج.

Abstract

Objective: The present study was conducted in the province of Al- Najaf, for the period from April 2013 until November 2013 , which aims to identify the *Entamoeba histolytica* parasites by screening for *Actin* and *Amebapore – A* genes in stool samples by using the real-time PCR technique.

Methodology: The study included , collect (104) stool samples from patients with diarrhea arrivals to Al-Sadar Medical City Hospital ,Al- Zahra Maternity and Children Hospital , Al-Manathera hospital and Al-Sajjad Hospital.

Results: The results of the real-time PCR revealed , the presence of *E. histolytica* parasites in ((25) stool samples out of the (104) samples, the rate (24%) .

Conclusion: In light of the results obtained through this study we concludes that the efficiency of real-time -PCR technique large and rapid in diagnosis of *E. histolytica* parasites in stool samples.

Recommendation: The study of gene expression of *Actin* and *Amebapore – A* genes, specific for *E. histolytica* parasite.

Keywords: Real-Time PCR, *Entamoeba histolytica*, *Actin*, *Amebapore – A*

INTRODUCTION

Amebiasis caused by *Entamoeba histolytica* is still mentioned as one of the major health problems in tropical and subtropical areas (1). It is the cause of various infectious diseases ranging from dysentery to abscess of liver or other organs.

It is estimated that amebiasis is responsible for up to 110,000 deaths per year (2 , 3) . This infection is usually predominant in low socioeconomic status and poor hygienic situations that favor the indirect fecal-oral transmission of the infection (4). Previously two morphologically identical species of *Entamoeba* had been found, and was shown that only one of them is able to cause infection in kittens or human volunteers (5). However, *E. histolytica* has recently been re-described as two distinct species; the pathogenic species *E. histolytica* and the nonpathogenic species *E. dispar*. As these two species are morphologically similar, development of new methods for their rapid differentiation is currently under investigation (6).

Actin is a cytoskeletal protein involved in cellular functions like maintaining cell morphology, cell motility and division, and intracellular transport (7). *Actin* proteins are encoded by a multigene family. In *Entamoeba histolytica*, two to four *Actin* genes have been described (8).

One of the remarkable capacities of *Entamoeba histolytica* trophozoites is their ability to lyse eukaryotic cells on contact. This ability derives from *Amoebapores*, a family of three pore-forming peptides (*Amoebapore A* [*AP-A*], *AP-B*, and *AP-C*) (9 , 10). *Amoebapores* insert into the membranes of bacteria or eukaryotic cells and form pores that result in lysis of the target cells. The addition of purified *Amoebapores* to eukaryotic cells results in cell necrosis and possibly apoptosis (11). Amplification of ameba DNA fragments by PCR has been proven to constitute a sensitive and specific method to detect *E. histolytica* or *E. dispar* from human feces (11 , 12).The present study was aimed to identification of *E.histolytica* parasite by detection and characterization of *Actin* and *Amebapore-A* (*AP-A*) genes in stool samples by using real-time PCR techniques.

MATERIALS AND METHODS

The present study was conducted for the period from April 2013 until November 2013, where he was collecting (104) stool samples from patients suffering from diarrhea, which is between the ages of (1-58) years who arrivals to some hospitals of Al-Najaf province (Al-Sadar Medical City Hospital, Al- Zahra Maternity and Children Hospital , Al-Manathera hospital and Al-Sajjad Hospital), where placed the samples in sterile plastic containers and record them some necessary informations such as the patient's name, age, residence, general appearance of the stool, and the presence of blood, and the presence of mucus, and preserved the samples in the temperature (-20°C) until used in the DNA extraction process **.**

Extraction of DNA from stool samples

DNA was extracted from stool samples according to (Bioneer Kit, Korea). Agarose gel electrophoresis was carried out to detect the presence of DNA (13).

Amplification by real-time PCR

Table (1):show the sequence of primers, which used for amplification of *Actin* and *Ap-A* genes for detection of *E. histolytica,* whereas the real-time PCR mixture was prepared as explained in table (2).

Table (1): Primers used for real-time PCR

Primer	DNA Sequence	Reference

AP- A	**F**	**5′CACTAAGGGAGCTGATAAAGTAAAAGATTA-3′**	**(14)**
	R	**5′-TCCAAAATCAAGAACTTTAGTGCAA-3′**	
Actin	**F**	**5′TGTAGATAATGGATCAGGAATGTGTAAA3′**	
	R	**5′-CAATGGATGGGAATACAGCTCTT-3′**	

Table (2):The mixture of real – time PCR

Content	Volume (μ l)
master mix with Sybr green	**20**
F- Primer (10 pmole)	**2**
R- Primer (10 pmole)	**2**
DNA Template	**10**
DEPC-distlled water	**16**
Total	**50**

Real-Time PCR protocol was carried out as following :denaturation at 95 ^{0}C for 5 minute and 45 cycle of 95 ^{0}C for 20 second , annealing at 55 ^{0}C for 40 second for *Actin* and 60 ^{0}C for 40 second for *AP-A*.Melting at 50-95 ^{0}C for 45 minute.

Binding of Sybr green to the real-time PCR product

Syber green is fluorescent dye and when it bind to double strand DNA , the fluorescence emission occurs which is used for the visualization of amplified product during Real-Time PCR of *Actin* and *Ap-A* genes with Sybr green .The amplification of each DNA specimen was determined by observation the fluorescence emission curve. These curves were produced due to binding Sybr green to the real-time PCR products, and the fluorescence reading were taken after each extension step of real-time PCR..The fluorescence emission increases due to the increase cycles of real-time PCR (15) .

MELTING CURVE ANALYSIS

After completion of (45) cycles PCR amplification , the PCR products were melted by raising the temperature from (50 Cº) to (95 Cº) at rate (1Cº/min.) **.**The Exicycler thermal block soft ware displayed the data collection during melting curve analysis as result melting temperature were derived from melting peaks by melting curve analysis of the amplified DNA specimens . Melting curve is of great importance, because the Sybr green will detect any double stranded DNA including primer dimers, contaminating DNA, and PCR product from misannealed primer. By viewing a dissociation curve, you ensure that the desired amplicon was detected (16).

RESULTS

The results of the real- time PCR showed that the amplified DNA for the genes *Actin* and *AP-A* in (25) stool samples out of (104) samples with the percentage (24%) by using specific primers and can be determined by a serving the fluorescence emission curves , the fluorescence curve which exceed the threshold line represent the amplified *Actin* and *AP-A* genes of *E. histolytica*, as shown in fig. (1) and (2).The negative samples did not amplified and the fluorescence curves remained below the threshold line as seen in fig. (3).

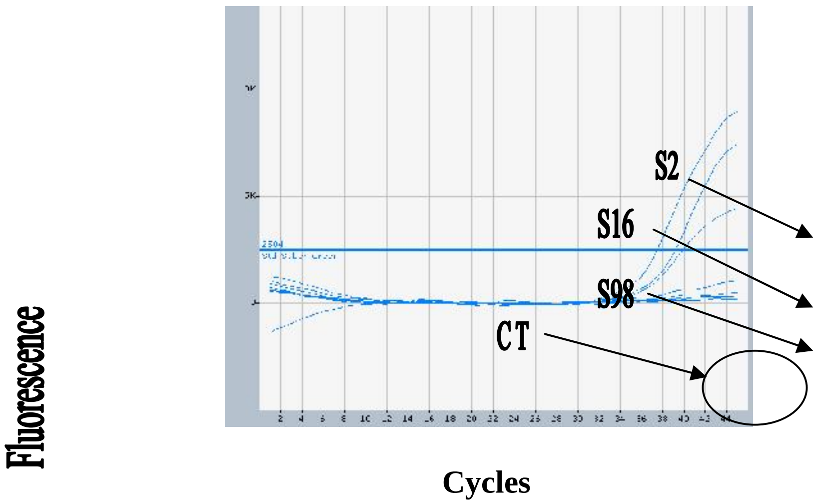

Fig.(1) Amplification of *Actin* gene of *E. histolytica* with Sybr green by using real –time PCR.
S= specimen, CT= Cycle Threshold
CT for S2=38.7 ; CT for S16 = 37.8 ; CT for S98 = 39.9

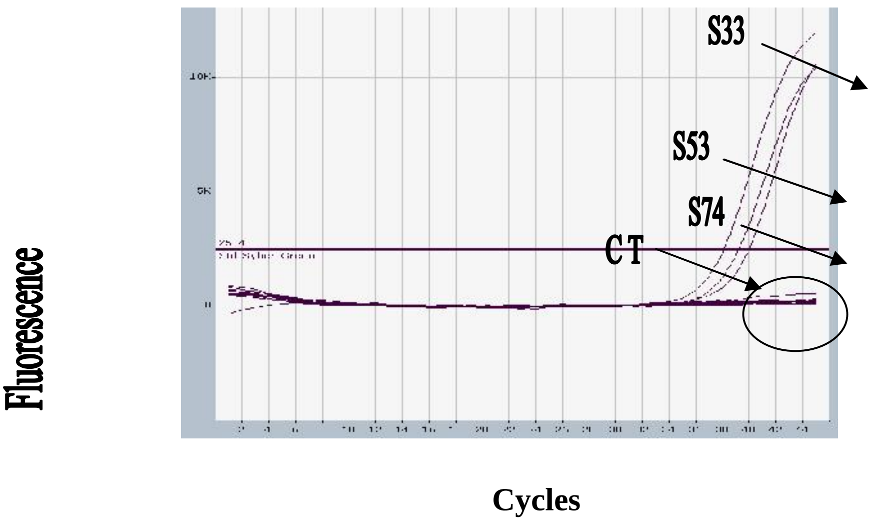

Fig.(2) : Amplification of *AP-A* gene of *E. histolytica* with Sybr green by using real- time PCR.
S= specimen, CT= Cycle Threshold
CT for S33 = 38.1 ; CT for S53= 39.3 ; CT for S74 = 40.0

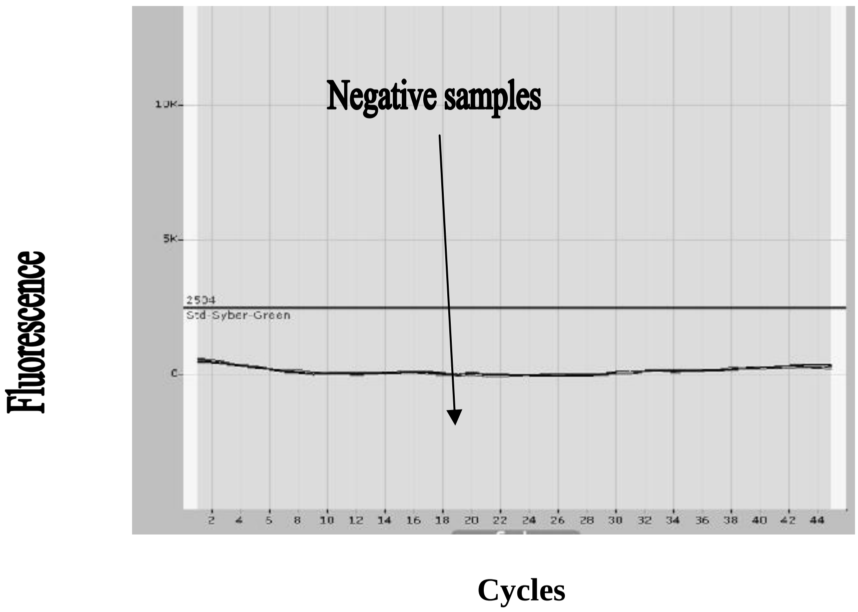

Fig.(3) Not amplified of *Actin* & *AP-A* genes of *E. histolytica* with Sybr green by using real –time PCR.

Melting curve

Melting curve analysis after completing the PCR. The temperature is then gradually increased and the fluorescence (syber green) is measured as function of temperature. The fluorescence decreases gradually with increasing temperature because of increased thermal motion which allows for more internal rotation in the bound dye. However, when the temperature is reached at which the double stranded DNA separates the dye comes off and the fluorescence drops abruptly this temperature, referred to as the melting temperature, as shown in fig.(4) &(5) .

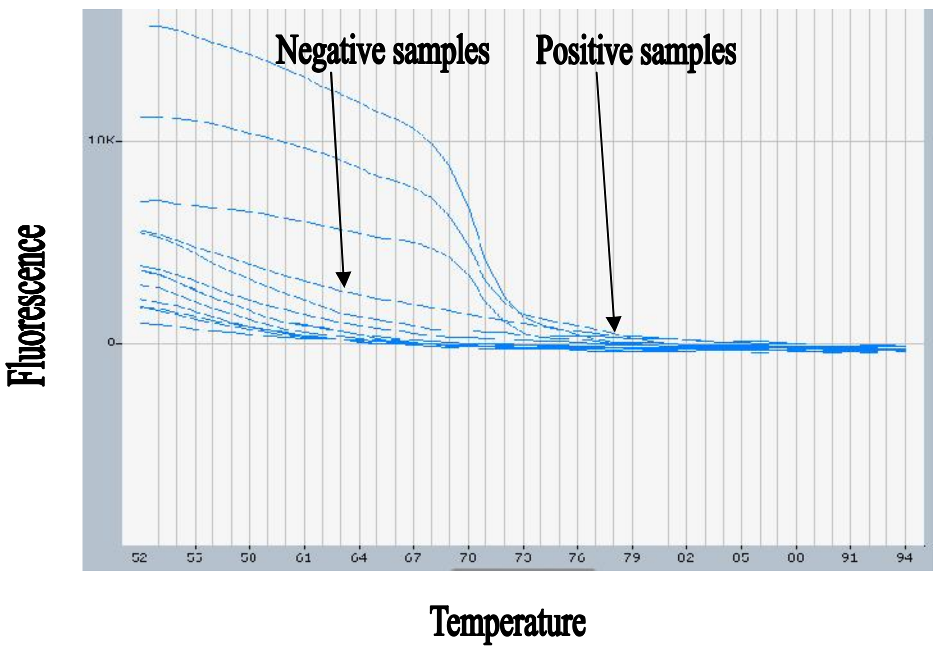

Fig.(4) The melting curve product for *Actin* gene

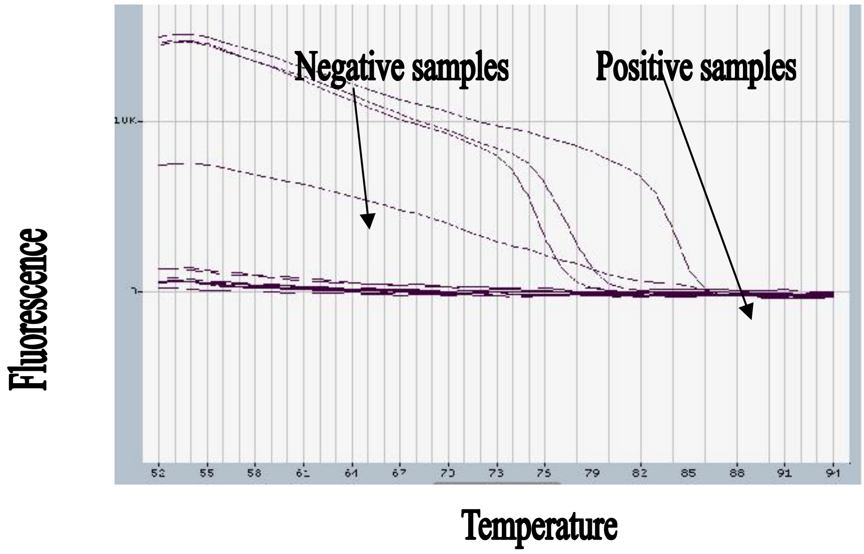

Fig.(5) The melting curve product for *AP*-A gene

DISCUSSION

Diagnosis of amoebiasis using molecular methods is useful not only in terms of diagnosis and but also for epidemiological studies through removing possible microscopy mistakes (17 , 18). Currently, *E. histolytica* antigen-specific ELISA or DNA determination (Real-Time PCR is more sensitive) tests are considered to be the most scientific alternatives for definitive diagnosis (19 , 20).

The results of the present study revealed that the amplified DNA by real-time PCR for *Actin a*nd *Amebapore-A* genes of *E.histolytica* in (25) samples out of (104) samples . The current study reinforced by (21), which found that the *Actin,* which occurs in the *E.histolytica* parasite encodes by two genes . DNA microarray screening also showed the upregulation of several genes encoding for proteins involved in *Actin* cytoskeleton dynamics during chemotaxis of *E.histolytica* towards Tumor Necrosis Factor (TNF) . These included proteins participating in microfilament dynamics, such as nucleation (Arp2/3, Formins), F-actin capping (Cap34α, Cap34β), microfilament network formation (ABP-120 (filamin), cortexellin), *Actin* bundling array (α actinin) (22)). Also the results of PCR showed The *E. dispar Actin* intergenic sequences differed in 83 nucleotides (22%) from those of *E. histolytica* (23) . There is abundant evidence that the actin cytoskeleton of the ameba is vital for adherence to target cells , cytotoxicity and phagocytosis process (24 , 25). On the other hand *Amebapore* genes are important in the detection of *E. histolytica* parasite and in turn these genes encode *Amebapore* enzymes which is of great importance in the pathogenesis of the parasite (26). The present study supported by (27), which found that *E. histolytica Amoebapores* exist as mature and potentially active peptides inside cytoplasmic granules of the trophozoite . The mode of action of the *Amoebapore* in viable *E. histolytica* trophozoites was that following the lectin-mediated recognition and intimate adherence between the amoeba and its target cell, the *Amoebapore* molecules are inserted into the membrane without depending on the interaction with a specific membrane receptor. As well as *Amoebapore* have antibacterial, cytotoxic and pore-forming activities (28) . Also *Amoebapore* expression is required for full virulence in the mouse model of amebic liver abscess, but *E. histolytica* trophozoites that do not express *Amoebapore-A* can still cause inflammation and tissue damage in infected human colonic xenografts (14).

CONCLUSION of the current study, it can be used *Actin* and *Amoebapore-A* genes in the detection of *E. histolytica* parasite by using real-time PCR..

RECOMMENDATION to study of the Gene Expression of *Actin* and *Amebapore – A* genes, specific for *E. histolytica* parasite.

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